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Specific and Rapid Enumeration of Viable but Nonculturable and Viable-Culturable Gram-Negative Bacteria by Using Flow Cytometry▽†

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An issue of critical concern in microbiology is the ability to detect viable but nonculturable (VBNC) and viable-culturable (VC) cells by methods other than existing approaches. Culture methods are selective and underestimate the real population, and other options (direct viable count and the double-staining method using epifluorescence microscopy and inhibitory substance-influenced molecular methods) are also biased and time-consuming. A rapid approach that reduces selectivity, decreases bias from sample storage and incubation, and reduces assay time is needed. Flow cytometry is a sensitive analytical technique that can rapidly monitor physiological states of bacteria. This report outlines a method to optimize staining protocols and the flow cytometer (FCM) instrument settings for the enumeration of VBNC and VC bacterial cells within 70 min. Experiments were performed using the FCM to quantify VBNC and VC *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, and *Salmonella enterica* serovar Typhimurium cells after staining with different fluorescent probes: SYTO 9, SYTO 13, SYTO 17, SYTO 40, and propidium iodide (PI). The FCM data were compared with those for specific standard nutrient agar to enumerate the number of cells in different states. By comparing results from cultures at late log phase, 1 to 64% of cells were nonculturable, 40 to 98% were culturable, and 0.7 to 4.5% had damaged cell membranes and were therefore theoretically dead. Data obtained using four different Gram-negative bacteria exposed to heat and stained with PI also illustrate the usefulness of the approach for the rapid and unbiased detection of dead versus live organisms.

Assessment of *Escherichia coli* O157:H7, *Pseudomonas aeruginosa*, *Pseudomonas syringae*, and *Salmonella enterica* serovar Typhimurium viability is a major requirement in several areas of microbiology, including public health, biotechnology, food technology, the water industry, and the pharmaceutical industry (32, 39, 43). A major concern related to culturing these bacteria is the inability to recover metabolically active, intact cells that have been exposed to environmental stresses, such as nutrient starvation, high or low temperatures, high pressure, and changes in pH or salinity (33, 43). Such bacteria are often defined as viable but nonculturable (VBNC) (9, 19, 23, 28, 32). The VBNC state is argued to be reversible because the cells, given the appropriate conditions, can be resuscitated to become culturable again (2, 14). For example, Kong et al. (21) showed that VBNC *Vibrio vulnificus* bacteria were culturable under anaerobic conditions. Moreover, pathogens in a VBNC state may remain virulent or produce enterotoxins (36, 40, 50). However, reports on true resuscitation of VBNC cells are very rare (3, 44), and substantiation of viability by other methods is complicated.

Novel methods to detect different states of bacteria in a range of environments are needed. New approaches should be rapid and accurate, so that results can be obtained within an hour or so and appropriate action can be taken. To offer an advantage over differential or selective culture methods, the innovative procedure should facilitate the detection and enumeration of specific viable bacteria. There has also been an increasing emphasis on the detection of specific organisms, particularly pathogens, rather than enumeration of indicator bacteria. Many existing techniques meet some, but not all, of these requirements. The traditional culture methods for detecting indicator and pathogenic bacteria in food and water may underestimate numbers due to sublethal environmental injury, inability of target bacteria to take up nutrient components in the medium, and other physiological factors which reduce culturability (18, 24, 26, 39, 51); however, these methods are also time-consuming (53–56) and cannot detect VBNC cells (8).

A large number of probes and methods enabling the physiological characterization of bacteria at the single-cell level have been developed recently (9, 17, 22, 46, 57). Most of these methods take more than 8 h and involve fluorescence-based methods; they include the direct viable count (DVC) method combined with nucleic acid staining (16, 20, 58), the double-staining method using epifluorescence microscopy (9), the measurement of respiratory activity with the fluorogenic dye 5-cyano-2,3-ditoyl tetrazolium chloride (38, 46, 48), the measurement of esterase activity with the ChemChrome fluorogenic substrate (35, 41), estimation of bacterial membrane potential using rhodamine 123 and fluorescein diacetate after 24 h of incubation (4), and the measurement of membrane

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integrity (5). LIVE/DEAD staining kits are widely used for viability assessment (19, 22); however, they are not universally applicable because SYTO 9, which is supposed to indicate live cells with intact membranes, can sometimes be preferentially excluded from some bacteria. Molecular methods based on PCR also have their drawbacks due to the effects of inhibitory substances (18) and take longer than the flow cytometer (FCM) methods (49, 57). Furthermore, the PCR assays alone cannot provide live/dead differentiation, although reverse transcription (RT)-PCR assays can overcome this limitation to some extent (11, 52).

The use of flow cytometry enables rapid, *in situ* analysis of single cells, including viruses (6), and in combination with staining techniques such as live/dead staining, quantitative as well as qualitative data can be obtained (39, 47, 53, 54). Multidimensional aggregation phenomena can be dynamically measured with an unmatched level of detail (42). Thousands of cells can be sorted and enumerated within a few seconds. Until now, flow cytometry has been used mainly in the field of clinical research (12). Detection and definition of bacterial viability (14, 29) have been the major microbiological issues in cytometry ever since the first paper on protein and nucleic acid synthesis in single organisms was published in 1975 (15). Using the FCM, it has previously been shown that four physiological states can be distinguished: reproductively viable, metabolically active, intact, and permeabilized (18, 30, 31), but rapid approaches to distinguish between VBNC and viable-culturable (VC) cells are not yet available.

During this study, it was assumed that cells having intact membranes are live and those with damaged membranes are dead or theoretically dead. Although there is considerable controversy surrounding the interpretation and verification of VBNC and VC cells, a simple calculation based on results from the FCM with paired dyes and standard nutrient media was used to determine the fractions of cells in different physiological conditions. Using different types of nucleic acid stains, FCM methods were developed to detect cells with intact and damaged membranes. In another instance, propidium iodide staining was used alone to detect the fraction of cells damaged during heat exposure. In both cases, the FCM settings were optimized to minimize noise and signals from detritus. Liquid counting reference beads were used to convert the FCM events to the number of cells per unit volume. Using this approach, methods can be developed for other bacterial species to efficiently identify and enumerate different physiological states of cells in a single-population sample. The main objective of this study was to establish the quickest, most accurate, and easiest ways to estimate the proportions of VBNC and VC states and dead cells, as indicated by membrane integrity of these four Gram-negative bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* O157:H7 (EPA strain 932), *Pseudomonas aeruginosa* strain PA01 from M. Franklin (Montana State University) (13), *Salmonella enterica* serovar Typhimurium SL3201 from B. A. D. Stocker (Department of Medical Microbiology, Stanford University, Stanford, CA), and *Pseudomonas syringae* (strain CC94, originally isolated from a plant leaf; obtained from INRA, Pathologie Vegetale, Montfavet, France) were used as model organisms.

E. coli O157:H7, *P. aeruginosa* and *S.* Typhimurium were grown separately on Luria-Bertani (LB) agar (Fisher Scientific, Palatine, IL) at 37°C for 20 h. *P. syringae* was grown on King's agar (medium A; components from Fisher Scientific, Palatine, IL) (1) at room temperature for 24 h. Single colonies from respective LB agar streak plates were transferred to three 50-ml culture tubes containing approximately 10 ml of LB broth (Fisher Scientific, Palatine, IL), and single colonies from King's agar were transferred to 50-ml culture tubes containing 20 ml of King's broth (King's medium A without agar; components from Fisher Scientific, Palatine, IL) (1).

Time to reach late log phase was determined for each organism in separate experiments (data not shown). The cultures in LB broth were incubated on a shaker at 180 rpm and 37°C for 18 h and were grown to late log phase at a concentration of 10^8 CFU/ml. The cultures in King's broth were incubated on a shaker at room temperature for 18 h. Culturability was examined by plating 50 to 100 μl of serial dilutions (10^{-2} to 10^{-6} in LB or King's broth) on LB or King's agar. The number of CFU (for culturable cells) per unit volume was determined in triplicate by plating 50 μl of serial dilutions on the respective agars.

Instruments and settings. The FCM (BD FACSAria, BD Biosciences, CA) used in this research was equipped with three lasers with excitation wave lengths of 407 nm (violet), 488 nm (blue), and 633 nm (red). Before running the cells in the FCM, adjustments of area scaling and quality control were done using rainbow beads (BD Biosciences, CA) according to the protocols provided by BD Biosciences, CA. The flow rate during area scaling adjustment and quality control was kept at 1.0, which was equivalent to 10 μl/min, and the number of events/s observed was 120 to 150. The window extensions of settings were kept at 2 μs. The sheath pressure was maintained at 70 lb/in^2 (high-pressure mode). The injection mode was set at high throughput, which was optimal for the protocols developed and used in this work.

Fluorescent probes and liquid counting beads. Initially, eight dyes that stain cells with either intact or damaged cell membranes were chosen. Four membrane-permeable dyes which stain live and dead cells (SYTO 9, SYTO 13, SYTO 17, and SYTO 40) and a membrane-impermeable DNA stain for cells with damaged membranes (propidium iodide [PI]) (Molecular Probes/Invitrogen, Eugene, OR) were ultimately selected for these organisms according to the highest degree of staining, best emission signals, fewest false-positive signals (coming from debris), and optimum number of events/s of cells displayed in the FCM histograms. The total cells (live and dead) were counted using the SYTO dyes, and the dead cells were enumerated using PI. Live cells (culturable and nonculturable cells) were calculated by subtracting the number of PI-stained cells from that of SYTO-stained cells. The nonculturable cell numbers were estimated by subtracting the number of plate count (culturable) cells from that of live cells.

The concentrations of SYTO 9, SYTO 13, SYTO 17, SYTO 40, and PI applied to the diluted pure cultures were as received (3.34, 5, 5, 5, and 20 mM, respectively) and applied at room temperature (~25°C). SYTO 9-, SYTO 13-, and PI-stained cells were excited by the blue laser. SYTO 17 and SYTO 40 were excited by red and violet lasers, respectively. The emission detectors used for the cells stained with SYTO 9, SYTO 13, SYTO 17, SYTO 40, and PI were 530 ± 15, 530 ± 15, 660 ± 10, 450 ± 20, and 576 ± 13 nm, respectively.

To convert the number of FCM recorded events of cells into concentrations, standard liquid counting beads (BD Biosciences, CA) were used. These beads were detected by the FCM in a separate region which did not correspond to the cells. Determined experimentally, the counting beads had optimal excitation and emission spectra of 488 and 620 ± 15 nm, respectively, which were detected using the same photomultiplier (PMT) used for SYTO 17. By running the liquid counting beads with bacterial cells and comparing the numbers of events detected in cell and bead regions, it was then possible to convert the number of events to the number of cells per unit volume. The bead concentration provided by the manufacturer for the lot used in this study was 947 beads/50 μl.

Sample preparation and flow cytometric analysis. During staining and enumeration, positive- and negative-control samples (with and without liquid counting beads, respectively) were used. The entire method was repeated with three or four separately grown cultures of each bacterium.

Initial tests were done to determine methods appropriate for reducing excessive electronic abort rates for the FCM. In a control study, cells at concentrations greater than ~10^4/ml caused high abort rates and concentrations less than ~10^4/ml resulted in interference with the electronic signal of the sheath fluid, which was subject to other false-positive signals. To ascertain that clumping did not occur, pure culture samples were vortexed for 2 min and diluted with the appropriate broth to obtain a cell number close to 10^4/ml. A total of 10 to 15 ml of diluted, stained cells was stained and passed through a sterilized 0.20-μm-pore-size and 25-mm-diameter hydrophilic membrane (GE Osmonics PCTE). The captured cells on the membrane were observed under the epifluorescence microscope, and cells were not clumped. These initial experiments confirmed that 10^4 cells/ml resulted in an abort rate close to zero (data not shown), which suggests good detection and minimal clumping of cells during counting using the

FCM. A specific amount of liquid counting beads in appropriate broth dilution media and Nanopure water that had been autoclaved and then filtered (0.1-μm-pore-size, sterilized mixed cellulose ester; Millipore) were run and assessed separately to identify the effect of noise, which typically arises from the presence of debris. These control experiments were conducted to determine the voltage and threshold settings, which indicate the signal level required to distinguish cells from debris; these settings are important control parameters required to establish an accurate protocol.

For flow cytometric analysis, the cells were diluted as described above. An aliquot was used to assess the number of CFU/ml in triplicate by plating 50 μl of serial dilutions on specific agar plates and under specific incubation conditions. In another 1-ml aliquot (number of cells, ~10^4/ml), 2.5 μl of each dye at received concentrations was added separately to individual samples, vortexed for 30 s, and incubated in the dark for 60 min. Initial experiments selected 1 h as the optimal staining time (data not shown). For each test condition, 1 ml of diluted stained samples was run without liquid counting beads in the FCM to optimize the setting and operating conditions (see Table S1 in the supplemental material), which took less than 3 min, and data were acquired. Then, depending on the number of events/s in the acquired data, 20 to 50 μl of liquid counting beads was added into a specific volume of stained sample and vortexed for 10 s. Usually for <500 events/s, 25 μl beads was added, but when the number of events/s was close to 1,000, 50 μl of beads was added. When the number of events/s was above 1,000, stained cells were diluted 1-fold with the appropriate broth to keep the abort rate close to zero. Finally within 2 min, the diluted stained sample with liquid counting beads was injected into the FCM, and data were acquired.

The total number of recorded events was 5,000 for cell counting. The optimized settings eliminated the overlapping of cells and beads and therefore allowed for the conversion of the number of events produced by bacteria to the number of cells based on the proportion of events per known number of beads. The amount of beads per volume was incorporated in the conversion of the number of events to that of cells.

The reproducibility of each protocol (optimized setting for each bacterium) was nearly 100%. Table S1 in the supplemental material gives the voltage settings and threshold parameters used while working with different dyes for different cells to obtain reproducible protocols. The cells were gated in forward (FW) versus side scatter (SS) plots. The total number of events in the cell region was obtained after creating a one-dimensional gate in the histogram for cells stained with a specific dye, and another one-dimensional grate was created on the liquid counting bead region displayed in the histogram. The number of events using each gate out of the total recorded 5,000 events was calculated. The following equation was applied to calculate the number of cells per unit volume, using the FCM.

$$\frac{\text{no. of events in cell region}}{\text{no. of events in bead region}} \times \frac{\text{no. of beads/test}}{\text{test volume}} \times \text{dilution factor} \quad (1)$$

The optimized setting for each bacterium was used to count different physiological states of cells in the log-phase cultures and cells with or without heat inactivation (described below). Data were acquired in three or four independent experiments.

Confirmation of gating protocols using cell sorting, DNA extraction, and agarose gels. Steps were taken to ensure that the selected protocols were able to differentiate between cells and debris. The histograms produced by the optimized methods typically were symmetric with small shoulders. To evaluate the minor populations represented by this shoulder in the histogram, they were also sorted separately from the main population comprising the rest of the histogram. Two one-dimensional gates were created for the populations in the shouldering of the histograms and also for the major population in the symmetrical or asymmetrical (skewed) distribution of histograms during the injection of stained cells after optimizing voltage setups and threshold values (see Table S1 in the supplemental material).

Diluted pure culture cells (8 ml at 10^4 cell/ml) were stained separately with different SYTO dyes, injected into the FCM, and sorted according to the respective optimized gated protocols for each dye and cell. The populations from single species stained with each dye displayed in these two gates were sorted in high-throughput mode and collected in two separate 5-ml sterilized culture tubes.

After sorting, the material in the tubes was vortexed and filtered separately through two sterilized 0.20-μm-pore-size and 25-mm-diameter hydrophilic membranes. To recover the remaining cells inside the tubes, the tubes were rinsed with filtered fluorescence-activated cell sorter (FACS) flow sheath fluid (BD Bioscience, CA) several times, vortexed, and filtered through the respective membrane consecutively.

These membranes were placed under the epifluorescence microscope to observe the abundance of cells sorted according to two gate regions using a 100× objective. As an additional test, the membranes with sorted cells were placed in PowerBead tubes from the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc., CA). The DNA was then extracted according to the manufacturer's protocols within 30 min after sorting. To confirm the existence and relative abundance of DNA from cells sorted by these two gates, an agarose gel was run for 30 min at 94 V. A 2% agarose gel solution was prepared by mixing 6 g of agarose powder (low-EEO/multipurpose/molecular biology-grade agarose; Fisher Scientific, IL) in 300 ml of 1× Tris-borate-EDTA (TBE) buffer (Invitrogen, Carlsbad, CA). The mixture was dissolved by heating, and finally 30 μl of 10-mg/ml ethidium bromide was added. After running the gel for 30 min, images were taken via a FluroChem IS-8800 (Alpha Innotech, CA). The extracted amount of DNA from the cells on the membranes is reflected in the thickness of the bands on the agarose gels and gives a relative estimation of the number of cells in each sorted sample.

Heat inactivation. Initial tests were done to select the best stain for evaluating membrane damage during heat inactivation of the four organisms. Three stains were used: PI, SYTOX blue, and 7-AAD (Molecular Probes/Invitrogen, Eugene, OR). All of these stains are believed to detect cells with damaged membranes. With the same heat-treated samples, different numbers of stained cells were observed microscopically and with the FCM (data not shown) with the three stains. PI was ultimately chosen to identify and enumerate the dead cells because it consistently gave the highest cell counts (data not shown).

Separate experiments also were done to confirm that exposure to PI did not affect the culturability of the four organisms before and after heat treatment using respective nutrient agars. An aliquot of cells in late log phase was exposed to PI, while a second aliquot was used as a control. Both aliquots were plated on the respective standard nutrient agar. No difference in counts was found (data not shown).

To determine if a simplified test for heat inactivation could be established, PI was used to detect fractions of heat-inactivated cells. One-milliliter aliquots of each pure culture cell in separate 2-ml sterilized culture tubes (RNase/DNase free; Fisher Scientific, IL) were placed in a 72°C heat block containing Nanopure water in each well, and the tubes were submersed into hot water for 5, 10, and 15 min. After heat exposure, the cells in suspension were brought to room temperature. Two culture tubes of each treatment of each heat-exposed cell were kept in an incubated shaker (same conditions used during pure culture of each type of cell) for 6 h. Cells that were or were not incubated after heat treatments and brought to room temperature were diluted and plated on the respective standard nutrient agars for culturable cell counts. The stained cells were incubated in the dark for 60 min and injected into the FCM. As necessary, the PI-stained cells were diluted with the appropriate broth for each organism when the abort rates were greater than zero. The same methods were used with cells that had not been exposed to heat, as controls. Based on the displayed number of events/s in the FCM, a known volume of control and heat-inactivated cells after PI staining was transferred into sterilized 5-ml culture tubes separately, and 20 to 50 μl of liquid counting beads was added in each tube and vortexed for 10 s prior to injecting them into the FCM. Two one-dimensional gates were created in the cell and bead regions in the respective histograms for each cell (either control or heat inactivated for different periods). Finally, the number of cells in each heat inactivation condition was calculated using equation 1.

The time required to reduce 90% of the culturable cells was estimated during heat treatment using nutrient plate count data and expressed as the decimal reduction time (*D* value). The standard errors of mean values were calculated from true independent replications (three or four separately grown cultures).

RESULTS

Selection of optimal dyes and enumeration of culturable and nonculturable cells. During initial studies, the incubation times of cells with different SYTO dyes varied from 10 to 60 min. After several trial-and-error approaches using the FCM and with the aid of an epifluorescence microscope (Eclipse E 800; Nikon, Japan), it was determined that the number of total SYTO-stained cells did not increase after a 30-min incubation period, except for *P. aeruginosa*, which required an additional 30 min of staining time for an unchanged total cell count to be recorded. Therefore, a 60-min incubation period was chosen for the method. For *E. coli* O157:H7, SYTO 9 and SYTO 17

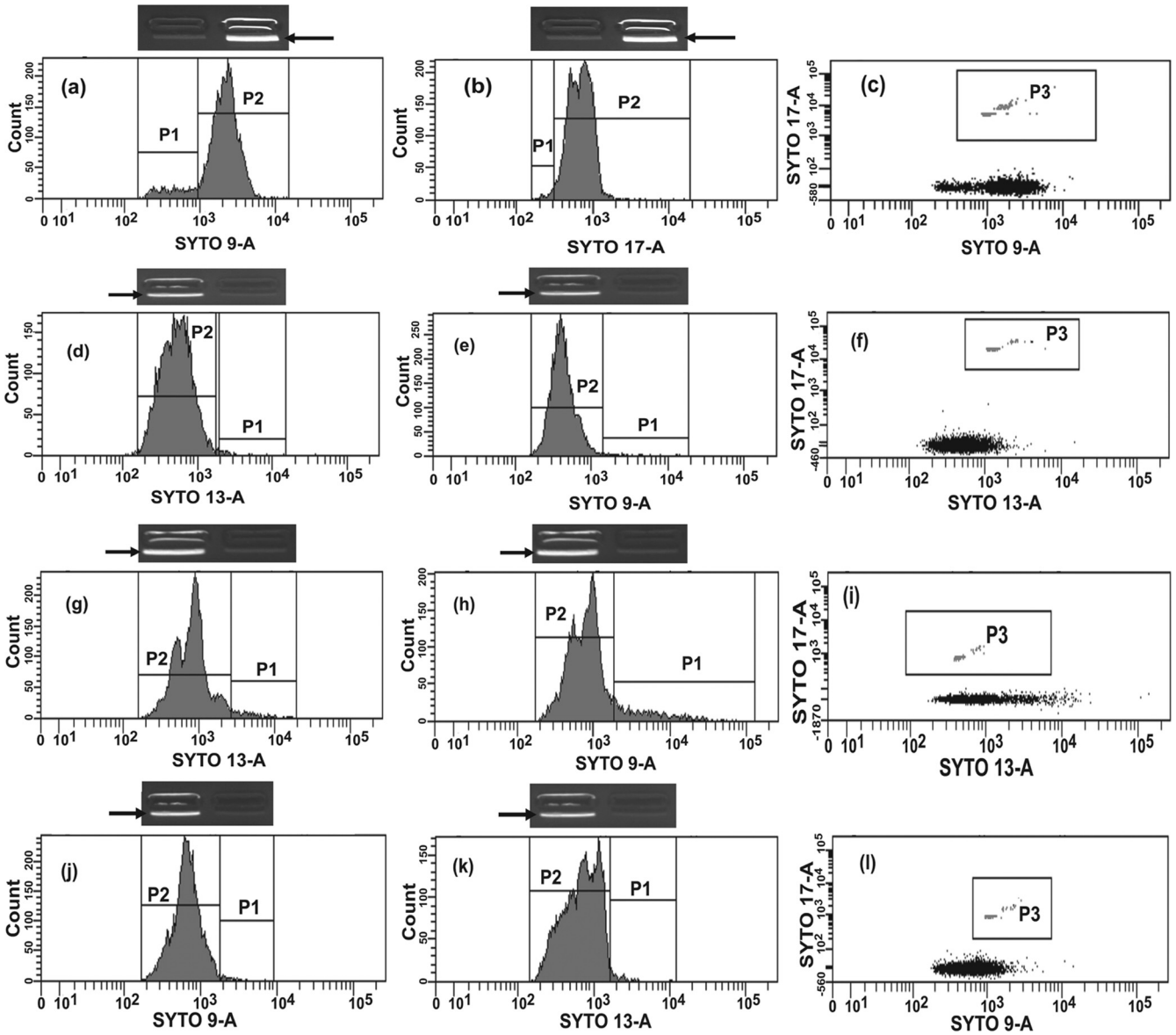

FIG. 1. Histograms of the most suitable dyes in terms of the highest culturable and nonculturable *E. coli* O157:H7 (a and b), *P. aeruginosa* (d and e), *P. syringae* (g and h), and *S.* Typhimurium (j and k) counts without fluorescent beads. The agarose gel images of extracted DNA from the populations in selected gates P1 and P2 are shown on the top of corresponding gates in the histograms. The arrow indicates the location of the DNA band in the agarose gel image. The dots in gate P3 (c, f, i, and l) show the liquid counting beads for each organism.

were found to be the most suitable dyes in terms of obtaining the highest number of viable and nonculturable cell counts, and for *P. aeruginosa*, *P. syringae*, and *S.* Typhimurium, SYTO 9 and SYTO 13 were found to be the most suitable. The one-dimensional gates, P1 and P2, were created, and cells in these regions were sorted using the high-throughput mode (Fig. 1). The counting beads injected into the FCM with respective stained species are shown in gate P3 (Fig. 1c, f, i, and l).

To confirm the optimized FCM settings, the shoulders in gate P1 and the events contributing to the main histogram in gate P2 were sorted separately, and sorted samples were passed through 0.20-μm-pore-size, 25-mm-diameter hydrophilic membranes. This was done to determine if the shoulders were unstained cells or debris. The dispersed stained cells were confirmed under the epifluorescence microscope and by the relative abundance of DNA extracted from cells in those gates on an agarose gel (Fig. 1). The agarose gel bands on the top of the histograms correspond to the abundance of sorted cells in those two regions. The material in the shoulder region (gate P1) was narrow and dim. In contrast, the extracted DNA from material sorted from the main histogram (gate P2) provided thicker and brighter bands, which corresponds to the abundance of cells in that region. This provides evidence that the sorting resulted in adequate separation of cells from debris, with band thickness and brightness directly proportional to the materials sorted from these regions and also to histogram width and peak intensity.

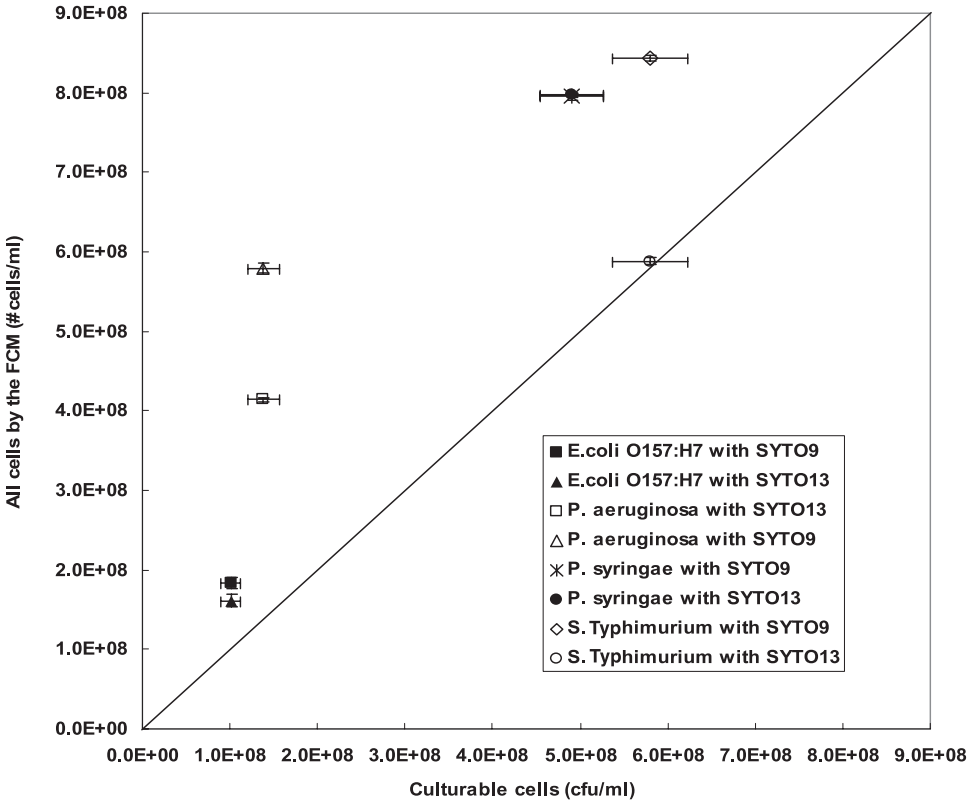

FIG. 2. Cell counts using the FCM for *E. coli* O157:H7, *P. aeruginosa*, *P. syringae*, and *S.* Typhimurium stained with the best two dyes, SYTO 9 and SYTO 13, versus culturable cell numbers using standard nutrient agars. The error bars along the *x* and *y* axes indicate the standard error of mean values of the respective measurements.

The histograms for SYTO 9- and SYTO 17-stained *E. coli* O157:H7 are shown in Fig. 1a and b, respectively. Using these dyes, more than 36% of cells from the log phase broth cultures were nonculturable, and close to 4% of cells were dead (see Table S2 in the supplemental material). For *P. aeruginosa* (Fig. 1d and e), the multiple peaks indicate that cells were stained with SYTO 13 and SYTO 9 while undergoing cell division. Even in pure culture, 63 to 74% of total cells were nonculturable and more than 2% of cells were dead (see Table S2). In the case of *P. syringae* (Fig. 1g and h) stained with SYTO 13 and SYTO 9, 36 to 37% of total cells were nonculturable and a lower percentage (<2%) of cells were dead (see Table S2). Figure 1j and k show the histograms for *S.* Typhimurium stained with SYTO 9 and SYTO 13 dyes. In this case, more culturable cells (69 to 99%) were identified and the proportion of dead cells was <1% (see Table S2). The differences between the percentages of culturable and nonculturable *S.* Typhimurium cells in the cases of SYTO 9 and SYTO 13 are significant and are due to the effect of membrane permeability of these dyes on *S.* Typhimurium.

By observing the separation between the cells and the beads, it was confirmed that these optimized settings resulted in isolated cell and bead regions. Using these data, the number of total stained cells (see Table S2 in the supplemental material) was calculated using equation 1. Note that there is an anomaly in the data when SYTO 40 was used, and a negative value for nonculturable cells was found. These data are an illustration of how optimal combinations of stains and instrument settings for each organism were obtained. Based on the results from the optimal dyes, 32 to 41% of cells were nonculturable and around 4% cells were dead. Therefore, even under ideal laboratory conditions in pure culture, only 60% of cells were cultured.

The numbers of CFU of culturable cells using the standard nutrient agars versus all cell counts using the FCM for these four bacteria stained with the best two dyes, SYTO 9 and SYTO 13, are shown in Fig. 2. The data points lie above the line of equality, which indicates that the FCM counts were higher than the plate count data. The culturable cell counts and the FCM count of *P. syringae* overlapped because the numbers of cells using the FCM after staining with SYTO 9 and SYTO 13 were similar. The variability of true mean values of the FCM counts reside over the line of equality within the narrow range of distribution, with a greater distribution for the culturable counts. This suggests that the FCM count was more repeatable and had higher precision than the plate counts. It is also evident that different stains provided different counts and therefore affected the ratios of VBNC to VC cells at certain physiological states. This illustrates the need to optimize dye selection for each species to achieve maximum counts.

Observation of membrane integrity after heat shock. Figure 3 illustrates the number of dead cells (measured with the FCM using PI staining) after each species was exposed to heat shock at 72°C for 0, 5, 10, and 15 min. The numbers of PI-positive *P.*

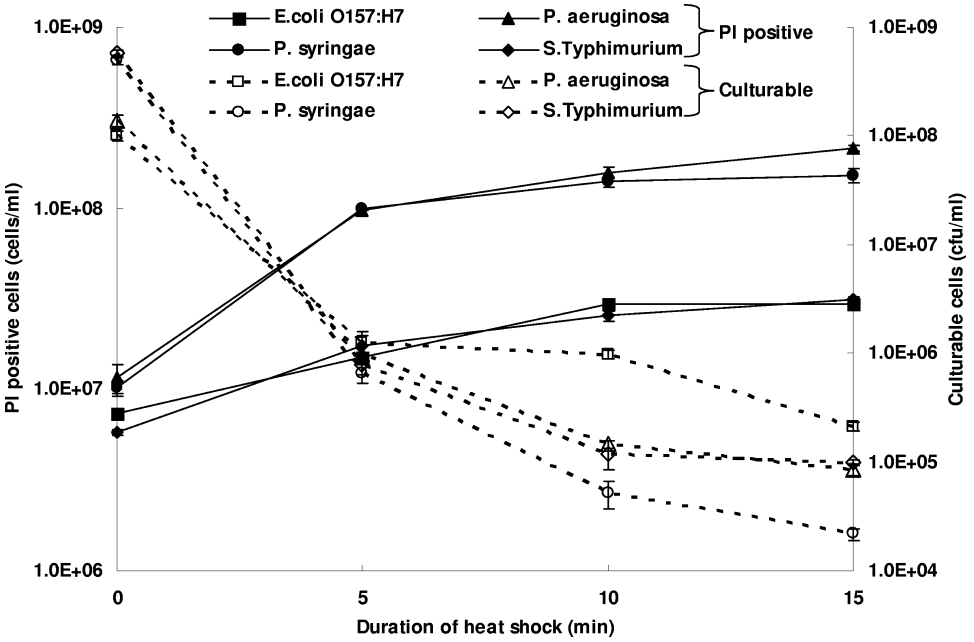

FIG. 3. The number of propidium iodide-positive cells (cells with damaged cell membranes or theoretically dead) and culturable cells after heat shock at 72°C for 0, 5, 10, and 15 min. The error bars are ± the standard error of means calculated from true independent replications (three or four separately grown cultures).

aeruginosa and *P. syringae* cells increased more rapidly than those of *E. coli* O157:H7 and *S.* Typhimurium cells during the first 5 min of heat inactivation, but this rate did not continue after this point (Fig. 3). Because PI can enter into the cells with compromised (damaged) membranes, a higher number of PI-positive cells represent more cell damage. The duration of heat shock required to produce damaged cell membranes was greater for *E. coli* O157:H7 and *S.* Typhimurium cells.

The number of culturable cells (measured on standard nutrient agar) during heat shock is shown in Fig. 3. With an increase in the duration of heat exposure, the cells lost culturability, and this rate decreased with time. The loss of culturability was very rapid (more than 2 logs) during the first 5 min, which indicates that heat inactivation arrested culturability while causing less damage to the cell membranes. Membrane damage during heat inactivation was faster initially, which is correlated with the rapid decline in culturability. It is interesting to note that even though the percentage of PI-positive *P. aeruginosa* cells was the highest, the culturable cell number of this bacterium was not the lowest. This indicates that cell membrane damage does not directly depend on cell culturability. The PI-positive *E. coli* O157:H7 and *S.* Typhimurium cell numbers were the same at 15 min, but their culturability levels at this point were not the same (1/2-log difference). Therefore, each type of cell, even from the same species, shows different relationships between cell membrane damage and culturability during heat exposure. The decimal reduction times (*D* values) of these organisms were less than 5 min, which were the first measured data after heat exposure without further incubations.

Figure S1 in the supplemental material shows representative histograms of PI-stained *E. coli* O157:H7 (Fig. S1a and b), *P. aeruginosa* (Fig. S1c and d), *P. syringae* (Fig. S1e and f), and *S.* Typhimurium (Fig. S1g and h) cells exposed to heat shock for 0 min and 5 min without further incubation. The cells and the counting reference beads are shown in the one-dimensional gates P1 and P5, respectively. The emission wavelengths of PI-positive cells and counting reference beads are completely separate, so the histograms in gates P1 and P5 did not overlap. With increased duration of heat exposure, the intensity and height of peaks increased, and this was quite noticeable in the first 5 min. However, the peak intensities and heights did not change substantially after 5 min of heat exposure.

DISCUSSION

Direct analysis of bacteria on a single-cell level will continue to enhance our understanding of microbial populations and their heterogeneity and complexity. This research was carried out to find an easier, more accurate, and much faster way to identify VBNC and VC cells and assess cell membrane integrity. The four Gram-negative model organisms used in this study are common targets in food technology, the water industry, and other areas of microbiology, emphasizing the importance of reliable enumeration in different physiological states. The numbers of total (after staining with SYTO dyes) and dead (after staining with PI) cells in laboratory broth cultures during log phase were determined using the FCM, and the culturable cell numbers were obtained using specific standard nutrient agars. These three values were used to calculate the VBNC and VC cells (see Table S2 in the supplemental material). The suggested ranking of dyes for each bacterium was based on the highest numbers of culturable and nonculturable cells detected by the FCM. The techniques developed in this study identified these physiological states accurately and quickly. The time required to analyze and count the cells using

the FCM was less than 10 min after 60-min incubation of cells with dyes. Thus, a sample can be prepared and enumerated within 65 to 70 min. The protocols established in this study using nutrient media and pure cultures were free from background noise, which is a common problem for the FCM.

One of the issues associated with the use of FCM is interference from background detritus, which can affect sensitivity. The optimized settings developed through this study eliminated the interference of other particles or debris in the cell enumerations. For example, a cell concentration of $\sim 10^4$/ml was ideal for detecting all of the organisms used in this study. During optimization of the methods, *E. coli* O157:H7 could be counted at levels as low as $\sim 10^2$/ml without any interference; however, the method was not as sensitive with the other organisms (data now shown). Because we proposed a generalized protocol, the cell concentration at $\sim 10^4$/ml was optimal. There could be other methods applied to improve sensitivity that went beyond the scope of this project. For example, numerous (or even rare) microbial cells could be detected against a background of other bacteria or nonbacterial particles by combining FCM with specific, fluorescently labeled antibodies or oligonucleotides probes (10, 14).

Application of the proposed method required the optimization of staining. One important consideration was the use of dyes that were retained by all cells (the SYTO dyes) versus another stain (propidium iodide [PI]) that labeled only those with compromised membranes. In this study, each sample of cells was stained with a single dye to avoid the potential of overlapping emission spectra. Although it is also common to establish a threshold on either side scatter (SS) or specific photomultipliers (PMT) for bacterial cells, the method described here reduced the interference of debris during cell counting and sorting. It was also found that there is no universal dye to stain all species for enumeration, although either SYTO 9 or SYTO 13 was most productive for the species evaluated.

The second aspect was optimization based on membrane integrity, by the use of dye exclusion. Exclusion of PI was the method of choice for the heat exposure studies. Sauch et al. (45) found that PI staining was a suitable viability indicator for *Giardia muris* cysts and Gram-negative bacteria when they were inactivated by heat or by a quaternary ammonium compound (benzyldimethyltetradecyl ammonium [BTA]). Another advantage of PI is that it does not suffer from the problems of enzyme activity stains, which are a lack of enzyme activity, poor substrate uptake, and even active dye extrusion (30, 31). It is also possible that the process can be hampered by the uptake of breakdown products (28). Ethidium monoazide (EMA) can also be used to stain cells, and the application of the EMA-PCR method can also quantify the live and dead cells, but sorting of these two populations is not possible using this method for further analysis. Chang et al. (7) demonstrated that when EMA concentrations increased there was purported damage to viable cells. However, PI may not be appropriate in all situations. The potential limitations of PI do not negate the approaches used here, but they do illustrate that optimization of stains is required.

It is known that bacterial cultures contain dead and VBNC cells; however, the proportion of live, dead, VBNC, and VC cells in samples has not been estimated previously. With log-phase broth cultures and using SYTO stains, this study showed that more than 30% cells were nonculturable, illustrating that viable cell numbers are underestimated with standard nutrient agar media, even under ideal conditions. In pure culture conditions, 0.7 to 4.5% cells were found to be dead, which indicates that the growth of cells depends not only on the nutrient conditions and optimum temperature but also on other factors. Furthermore, the heat-treated cells were transferred to the incubated shaker for 6 h and then plated on the respective standard nutrient agars for culturable cell counts. It was observed that 1 to 4% of nonculturable cells after heat shock became culturable under these conditions. It has been argued that nonculturability of cells can be reversible (2, 14, 23, 37, 43), and this is borne out by the present study. These values should not be taken as absolutes since the culturability or entry into the VBNC state by different bacteria will vary from one species to another (34) and also depends on the media and other growth conditions.

Further evaluations were done with heat treatment and PI because heat treatment is the most common method of food preservation and causes a significant reduction in bacterial cell numbers (27, 31). The correct application of heat is critical to render a product safe or to achieve a required extended shelf life. The critical parameters of the process are the absolute temperature and the period of treatment. While heat transfer in food materials is inherently very complex, bacterial heat injury simulation studies can readily be carried out in a temperature-controlled water bath. In this study, the FCM enumeration was more sensitive than nutrient agar media. Moreover, by definition, the agar media cannot detect VBNC cells. Murphy et al. (27) reported that the *D* values for *E. coli* O157:H7 and *Salmonella* were less than 5 s at 70°C. However, the complete loss of culturability of cells in our study was not achieved during 15 min of heat treatment at 72°C, which could be an indication of membrane integrity of these organisms. Standard nutrient media were used for this study; however, application of specific media, plating method (pour plate versus spread plate), incubation period, temperature, etc., for each bacterium will affect the enumerations of VBNC and VC cells.

Different chemical disinfectants used in many potable waters may cause sublethal injury of some bacteria (25), thereby rendering them nonculturable. A potential application of this FCM-based method after optimization would be assessing the number of viable and culturable or nonculturable bacteria during disinfection. The methods developed in this study could be adapted for specific indicator organisms, thus providing a more rapid and sensitive method for assessing the regulatory bacteriological quality of water or the effectiveness of disinfection.

For any other systems, the approach outlined herein can be applied to other organisms, although modest modifications in dilution media and incubation periods with stains may be required for optimization. The premise was that different organisms would be optimally stained with some but not all dyes because the nucleic acid binding capacities of these dyes differ. The most challenging aspects of using the FCM for detecting cells with compromised membranes are the selection and optimum excitation of the best dye; this report details how these can be done in a systematic manner. Optimized flow cytometry methods show promise for the rapid, unbiased detection of

portions of bacterial populations that are alive with intact membranes and theoretically dead with compromised membranes. The optimized methods for dye selection and noise reduction as described herein can be used to assess the response of organisms to environmental stress. The extension of this study will be to identify VBNC and VC cells in a mixed culture in the presence and/or absence of foreign particles.

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